Chemical Mechanism of the Reaction Catalyzed by Dihydrofolate Reductase from *Streptococcus faecium*: pH Studies and Chemical Modification†

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ABSTRACT: The variation with pH of the kinetic parameters associated with dihydrofolate reductase from *Streptococcus faecium* has been used to gain information about the chemical mechanism of the reaction catalyzed by the enzyme. The pH dependence of log V/K for dihydrofolate showed that a group with a pK value of 4.7 must be ionized and that a group with a pK value of 6.6 must be protonated for activity. Temperature and solvent perturbation studies indicate that these groups are probably the carboxyls of the glutamate moiety of dihydrofolate and of an aspartate residue on the enzyme, respectively. The similarity of the pH profile and the magnitude of the pK value for the linear competitive inhibitor 2,4-diaminopteridine suggest that the carboxyl group is concerned with the binding of dihydrofolate and its analogues to the enzyme. This conclusion is confirmed by the result that a group with a pK value of 6.7 must be protonated for the binding of methotrexate. It is proposed that the binding involves the formation with N-5 of dihydrofolate or N-1 of methotrexate of a hydrogen bond which has considerable ionic character and which lies within a hydrophobic environment. Further, it is suggested that the same hydrogen acts as an auxiliary catalyst which facilitates hydride transfer from NADPH to dihydrofolate for its conversion to tetrahydrofolate. Evidence to support this suggestion comes from the finding that the V profile is similar to the V/K profile except that the pK of the group which must be protonated for maximum enzyme activity is shifted upward by about 2 pH units. Such an increase in a pK value is consistent with the formation of a hydrogen ionic bond in the ternary enzyme–NADPH–dihydrofolate complex. The results of inactivation experiments with trinitrobenzenesulfonate appear to indicate that a lysine residue is necessary to maintain the enzyme in its active conformation.

The enzyme dihydrofolate reductase (EC 1.5.1.3), which catalyzes the reaction 7,8-dihydrofolate + NADPH + H^+ → 5,6,7,8-tetrahydrofolate + NADP, occupies a key position in the biosynthesis of DNA. Hence, it has been subjected to extensive investigation, especially with regard to its inhibition by antifolate drugs (Blakley, 1969; Williams et al., 1980). Little attention has been paid to the determination of the chemical mechanism of the reaction through the application of kinetic techniques although studies have been directed toward the elucidation of the kinetic mechanism (Burchall & Chan, 1969; McCullough et al., 1971; Blakley et al., 1971).

Information about the amino acid residues involved with catalysis has been deduced from X-ray crystallographic, NMR, and chemical modification studies. From the results of such studies, it has been concluded that arginine (Cocco et al., 1978; Vehar & Freisheim, 1976), aspartate (Matthews et al., 1978), histidine (Greenfield, 1974; Birdsall et al., 1977), lysine (Vehar et al., 1976), methionine (Gleisner & Blakley, 1975; Blakley et al., 1978), tyrosine, and tryptophan (Kimber et al., 1977) are present at the active site and are involved in catalysis.

Recently, Cleland (1977) has discussed the kinetic procedures for determining the amino acid residues at the active site of an enzyme which are involved with substrate binding and with catalysis. Thus, it was of interest to compare the conclusions reached from the application of pH studies with those deduced from the aforementioned investigations. The present results indicate that a protonated aspartate residue on the enzyme not only is invovled with the binding of dihydrofolate and folate analogues but also acts as an auxiliary catalyst for the reduction of the N-5 to C-6 double bond of dihydrofolate.

Materials and Methods

Materials

NADPH was purchased from P-L Biochemicals and folic acid from Calbiochem. Methotrexate was obtained from ICN Chemical and Radioisotope Division while 2,4-diaminopteridine and 6,7-diethyl-2,4-diaminopteridine were gifts from Dr. D. J. Brown. Diethyl pyrocarbonate was supplied by Pfaltz and Bauer Inc. and 2,4,6-trinitrobenzenesulfonate (TNBS) by Sigma. Dihydrofolate was prepared by reduction of folic acid according to the method of Blakley (1960) and stored at −20 °C for no longer than 2 weeks. Dihydrofolate reductase was isolated from a methotrexate-resistant strain of *Streptococcus faecium* A by the procedure of Williams et al. (1979).

Methods

Concentrations of methotrexate were determined at pH 13 and 302 nm by using a molar extinction coefficient of 22 100 (Seeger et al., 1949). The concentrations of dihydrofolate and NADPH were determined enzymatically by using dihydrofolate reductase and a molar extinction coefficient of 12 300 at 340 nm (Hillcoat et al., 1967).

Kinetic Assays. The activity of dihydrofolate reductase was determined spectrophotometrically at 340 nm and 30 °C as described previously (Williams et al., 1979). Control experiments showed that the enzyme was stable in both the absence and the presence of methoxyethanol over the range of pH values which was used and that the Michaelis constant for NADPH did not vary significantly between pH 4.5 and 9.0. At pH values below 6.0, there was a nonenzymatic change in absorbance which decreased as a linear function of time. This rate was subtracted from that obtained in the presence of the

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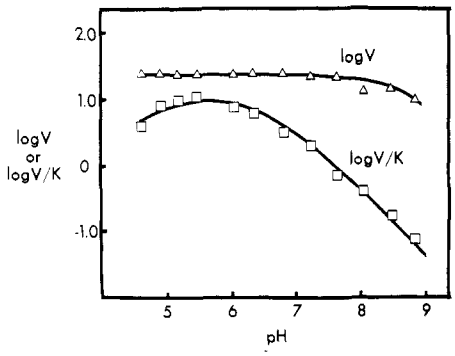

FIGURE 1: V/K for dihydrofolate and V profiles for the dihydrofolate reductase reaction.

enzyme. Progress curves in the presence of methotrexate were obtained as described by Williams et al. (1979).

Since the activity of dihydrofolate reductase is sensitive to changes in ionic strength (Nixon & Blakley, 1968), it was necessary to ensure that the ionic strength of reaction mixtures did not change as a function of pH. This objective was achieved through the use of a computer program for calculating the ionic strength of mixtures of two or three buffers at a given pH and temperature (K. J. Ellis and J. F. Morrison, unpublished experiments). With a buffer mixture containing 2-(*N*-morpholino)ethanesulfonic acid (Mes, 0.025 M), sodium acetate (0.025 M), tris(hydroxymethyl)aminomethane (Tris, 0.05 M), and NaCl (0.1 M), the ionic strength remained constant at an optimum value of 0.15 over the range from pH 4.0 to pH 9.0.

Solvent perturbation studies with 20% (v/v) 2-methoxyethanol were performed in a neutral acid buffer mixture containing sodium acetate (0.025 M), maleic acid (0.025 M), and dimethylmalonic acid (0.025 M) as well as in a cationic acid buffer mixture containing Mes (0.025 M) and *N*-2-(hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes, 0.025 M). The ionic strength was calculated as described above for each buffer mixture at each pH, and NaCl was added to ensure that the ionic strength remained constant. For studies in the presence of 2-methoxyethanol, the organic solvent was added after determination of the pH of the assay mixture. All pH determinations were made with a Radiometer 26 pH meter standardized at the given temperature to ±0.01 pH unit with Beckman buffers.

Chemical Modification of the Enzyme. Dihydrofolate reductase was modified by using TNBS or freshly prepared diethyl pyrocarbonate. Unless otherwise stated, reactions were performed at 20 °C in the presence of 0.05 M sodium phosphate–0.15 M NaCl (pH 7.0) and with the modifying reagent at a final concentration of 5.0 mM. Modification of the enzyme was monitored continuously by following the change in absorbance at 267 nm with TNBS (Plapp et al., 1971) or at 242 nm with diethyl pyrocarbonate (Ovadi et al., 1967). Over the same period, samples of the reaction mixture were taken for assay of enzymatic activity. *N*-Carbethoxy groups were removed from histidine residues by incubating the modified enzyme with hydroxylamine (1.0 M, pH 7.0) for 30 min at 20 °C. Controls with unmodified enzyme were run simultaneously. Enzyme concentration was determined by fluorescence titration with methotrexate (Williams et al., 1979).

Progress Curves. Progress curves for the slow, tight-binding inhibition of dihydrofolate reductase by methotrexate were obtained and analyzed as described by Williams et al. (1979).

Analysis of Data. Values for V and V/K at each pH value were obtained by determining the steady-state velocity as a function of dihydrofolate concentration, at saturating levels of NADPH (90 μM), and then fitting the data to eq 1. Data

$$v = \frac{VA}{K + A} \tag{1}$$

conforming to linear competitive inhibition were fitted to eq 2 to obtain K_i values. For these purposes, the computer programs of Cleland (1979) were used. The pK values were

$$v = \frac{VA}{K(1 + I/K_i) + A} \tag{2}$$

obtained by fitting data for the pH profiles to either eq 3 or eq 4 by using the STEPIT computer program which was obtained from the Quantum Chemistry Program Exchange as part of the STEPT package (QCPE 307). Equation 3 was

$$\log y = \log\left(\frac{C}{1 + H/K_1 + K_2/H + K_2/K_1}\right) \tag{3}$$

$$\log y = \log\left(\frac{C}{1 + K_2/H}\right) \tag{4}$$

used when the pH profile was bell shaped. The interaction term, K_2/K_1, allows the direct determination of the true pK values irrespective of how close they may be to each other. Equation 4 was used to fit pH profile data when activity was lost on the basic side of the profile. In eq 3 and 4, y represents V, V/K, or $1/K_i$. Values for apparent first-order rate constants and for ΔH_{ion} were obtained by fitting data to eq 5 and 6, respectively.

$$\ln v = \ln v_0 - kt \tag{5}$$

$$\text{p}K = \frac{\Delta H_{ion}}{2.303RT} \tag{6}$$

Results

pH Profiles. The effect of pH on the reaction catalyzed by dihydrofolate reductase was determined by varying the dihydrofolate concentration at a fixed NADPH concentration (90 μM) which was at least 20 times its K_m value at any pH. The data of Figure 1 indicate that the pH profile for V differs considerably from that for V/K. Whereas the V profile shows a single break with loss of enzyme activity at high pH, the V/K profile is bell-shaped as a result of the loss of enzyme activity at both high and low pH. Analysis of the V profile data (eq 4) yielded a pK value of 8.6 ± 0.2 while analysis of the V/K profile data gave pK values of 4.73 ± 0.21 (pK_1) and 6.56 ± 0.12 (pK_2).

To elucidate the identity of the groups whose state of ionization affects the catalytic activity of the enzyme (Figure 1), we made determinations of the temperature dependence of the V/K profile and of the effects of an organic solvent on the pK values observed in neutral and cationic buffers. V/K profiles were obtained at 15, 21, and 35 °C. Each set of data was fitted to eq 3, and the resulting pK values were analyzed by using eq 6. The enthalpies of ionization (ΔH_{ion}) were −8.1 ± 7.3 (pK_1) and 11.3 ± 2.1 (pK_2) kcal/mol as calculated from the sets of lower and higher pK values, respectively. The effects on the pK values of adding methoxyethanol (20%) to reaction mixtures containing neutral or cationic buffers are illustrated in Table I. It will be noted that the organic solvent has no significant effect on the value of pK_1 in the presence of a neutral buffer. Such a result is consistent with the ionizing group being of the neutral acid type. Although there is an elevation of the pK_2 value by methoxyethanol in the presence

Table I: Effect of Methoxyethanol on the pK Values Determined from the V/K Profile for Dihydrofolate[a]

buffer type	− methoxyethanol		+ methoxyethanol	
	pK_1	pK_2	pK_1	pK_2
neutral[b]	5.04 ± 0.13	7.48 ± 0.16	4.82 ± 0.07	7.09 ± 0.05
cationic	[c]	6.77 ± 0.10	5.36 ± 0.07	7.38 ± 0.19

[a] Experiments were performed in the absence and presence of 20% methoxyethanol as described under Materials and Methods. [b] Neutral buffer contained sodium acetate (0.025 M), maleic acid (0.025 M), and dimethylmalonic acid (0.025 M) while the cationic buffer contained Mes (0.025 M) and Hepes (0.025 M). [c] Not determined.

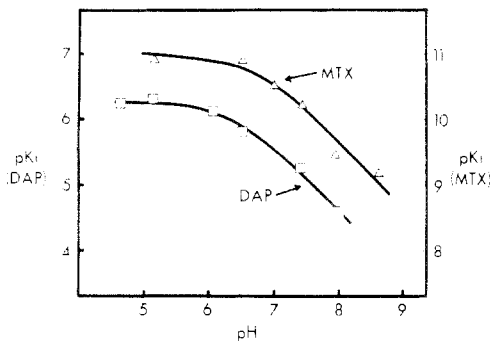

FIGURE 2: Variation with pH of the pK_i values for 2,4-diaminopteridine (DAP) and methotrexate (MTX). The pK_i values for methotrexate are for the overall inhibition constant which is equal to $K_i[k_r/(k_f + k_r)]$. The definition of the symbols is given in Table II.

of a neutral buffer, the increase is greater in the presence of a cationic buffer. Hence, it is concluded that the group associated with pK_2 is also a neutral acid. It should be pointed out that the value for pK_2 is significantly higher in a neutral acid buffer than in the buffer mixture used to obtain the data of Figure 1. Solvent perturbation experiments could not be used to determine the nature of the ionizing group associated with V. This was because of the higher magnitude of the Michaelis constant for dihydrofolate at alkaline pH and the high absorbance of the substrate at 340 nm which preclude the addition of high concentrations of dihydrofolate.

To determine if the ionizing groups observed in the V/K profile (Figure 1) are involved with the binding of substrate, we examined the pH dependence of the K_i value for 2,4-diaminopteridine (DAP), which functions as a linear competitive inhibitor with respect to dihydrofolate (Williams et al., 1980). The data (Figure 2) show that the break in the pK_i–pH profile on the basic side corresponds to that which is seen in the log (V/K)–pH profile (Figure 1). The similarity is emphasized further by the finding that the pK value of 6.31 ± 0.09 is not significantly different from that of 6.56 ± 0.12 for pK_2. Similar experiments were performed with methotrexate (MTX), which behaves as a slow, tight-binding inhibitor of the dihydrofolate reductase reaction (Williams t al., 1979). The inhibition involves the rapid formation of an enzyme–NADPH–MTX complex that undergoes a relatively slow, reversible isomerization reaction. From the quantitative analysis of progress curve data obtained at each pH, values were determined for the forward (k_f) and reverse (k_r) isomerization constants as well as for the dissociation constant for the rapid release of MTX from the enzyme–NADPH–MTX complex and for the overall inhibition constant (K_i*). The pH–pK_i* profile is illustrated in Figure 2. Analysis of these data by fitting to eq 4 yielded a pK value of 6.61 ± 0.09 which is similar to those obtained by using 2,4-diaminopteridine as an inhibitor (Figure 2) and from the log (V/K)–pH profile

Table II: Variation with pH of the Kinetic and Rate Constants Associated with the Inhibition of Dihydrofolate Reductase by Methotrexate[a]

pH	kinetic parameter[b]		
	K_i (nm)	k_f (min^{-1})	k_r (min^{-1})
5.14	2.4 ± 0.6	3.1 ± 0.9	0.015 ± 0.004
6.52	3.5 ± 0.8	3.8 ± 1.0	0.013 ± 0.002
7.00	11 ± 2	4.7 ± 0.8	0.013 ± 0.004
7.40	23 ± 8	5.1 ± 1.8	0.013 ± 0.001
7.95	27 ± 6	2.9 ± 0.8	0.037 ± 0.006
8.62	46 ± 17	2.6 ± 1.1	0.036 ± 0.007

[a] Experiments were performed and the data analyzed as described under Materials and Methods [cf. Williams et al. (1979)]. [b] K_i denotes the dissociation constant for the rapid combination of methotrexate with the enzyme–NADPH complex while k_f and k_r represent, respectively, the forward and reverse rate constants associated with the slow isomerization of the enzyme–NADPH–methotrexate complex.

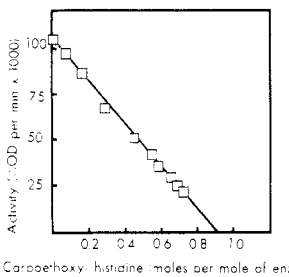

FIGURE 3: Relationship between the loss of dihydrofolate reductase activity and the modification of histidine residues with diethyl pyrocarbonate. Velocities were determined at 340 nm.

(Figure 1). It appears the change in the K_i* value as a function of pH is due solely to the variation of K_i with pH as the values for k_f and k_r are essentially pH independent (Table II). Although the pK_i–pH data could not be fitted to eq 4, computer simulation indicated that they were consistent with a pK value of 6.6.

Chemical Modification of Dihydrofolate Reductase. When the enzyme was reacted with diethyl pyrocarbonate (5.0 mM) at pH 7.0, as described under Methods, there was a 50% loss of activity within 10 min. Further incubation resulted in the complete loss of activity which corresponded to the modification of one histidine residue per mole of enzyme (Figure 3). There was no protection against enzyme inactivation by diethyl pyrocarbonate when 2,4-diaminopteridine (150 μM), either alone or with NADPH (10 μM), was present in reaction mixtures. Indeed, there appeared to be an increase in the rate of inactivation under each of these conditions. On the addition of hydroxylamine to the fully inactivated enzyme, there was no recovery of activity even though carbethoxy groups were removed as judged by the decrease in absorbance at 242 nm.

The enzyme is also inactivated by 2,4,6-trinitrobenzenesulfonic acid (TNBS), but at a rate which is 3 times slower than that with diethyl pyrocarbonate. Complete inactivation of the enzyme occurs on modification of three–four amino groups (Figure 4). Neither 6,7-diethyl-2,4-diaminopteridine (150 μM) nor NADPH (45 μM) plus diethyldiaminopteridine protect against inactivation. Instead, there is an acceleration of the rate of enzyme inactivation by TNBS with complete inactivation corresponding to the modification of one–two amino groups. The presence in the reaction mixture of NADPH (45 μM) alone resulted in an initial loss of 40% of the enzyme activity with modification of about one amino group. Subsequently, a further two amino groups were modified without any further loss of enzyme activity. The

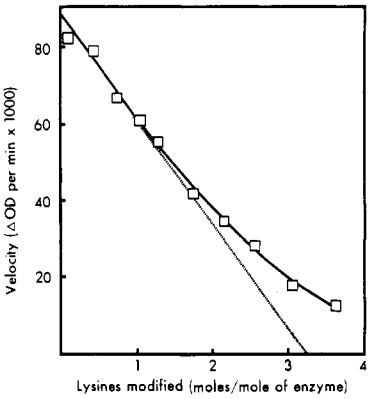

FIGURE 4: Determination of the number of lysine residues that must be modified by trinitrobenzenesulfonate for complete inactivation of dihydrofolate reductase. Velocities were determined at 340 nm.

inactivation of the enzyme by TNBS is a pseudo-first-order reaction from pH 7.0 to 9.0 with k_{obsd} increasing as the pH is raised [cf. Freedman & Radda (1968)].

Discussion

Histidine has been implicated as the acid–base catalyst which is required for a variety of dehydrogenase reactions (Dalziel, 1975). From the inactivation of dihydrofolate reductase by diethyl pyrocarbonate, which is considered to be reasonably specific for reaction with histidine residues (Ovadi et al., 1967), and the formation of one carbethoxyhistidine per mole of enzyme (Figure 3), it might be concluded that a histidine residue was involved with the conversion of dihydrofolate to tetrahydrofolate. However, 2,4-diaminopteridine with or without NADPH did not protect against enzyme inactivation, and there was no reactivation of the modified enzyme by hydroxylamine even though the carbethoxy groups were removed. This modification of a histidine residue is either not responsible or not solely responsible for loss of catalytic activity. In this connection, it is of interest to note that there is no histidine residue at the active site of enzymes from *Escherichia coli* and *Lactobacillus casei* (Mathews et al., 1978) and that other amino acid residues can undergo modification with diethyl pyrocarbonate (Burstein et al., 1974). Inactivation was also obtained by treatment of dihydrofolate reductase with trinitrobenzenesulfonate, and as the enzyme from *S. faecium* does not contain cysteine residues, such inactivation must be due to modification of amino groups. Although complete loss of activity is achieved in the absence of ligands by modification of three–four amino groups, the presence of NADPH prevents complete loss of activity while with NADPH plus 2,4-diaminopteridine there is acceleration of the rate of inactivation and complete loss of activity when one–two amino groups are modified. These results suggest that the lysine residues which undergo modification are not involved with the binding of substrate but may be required for a conformational change that is associated with catalysis.

The plot of log (V/K) for dihydrofolate as a function of pH shows breaks at both high and low pH (Figure 1). The break at low pH corresponds to a pK value of 4.73 and has a limiting slope of +1. Thus, a group in the substrate or in the enzyme–NADPH complex must be protonated for enzymatic activity. Since the γ-carboxyl group of the glutamyl moiety of dihydrofolate has pK value of 4.8, it seems likely that it is the ionization of this carboxyl group that is being observed on the low side of the V/K profile. This conclusion is supported by the finding that the ΔH_{ion} of the group (−8.1 ± 7.3 kcal/mol) is not significantly different from zero and by the fact that solvent perturbation does not cause a significant shift in the pK value when the profile is determined in the presence of a neutral buffer (Table I). It is also of interest to note that the ionized γ-carboxyl group of the glutamyl moiety of methotrexate reacts strongly with a cationic group on the enzyme which is probably an arginine residue (Matthews et al., 1977; Gleisner et al., 1974).

The break in the V/K profile at the higher pH (Figure 1) has a limiting slope of −1 and corresponds to a pK value of 6.56. As dihydrofolate does not contain an ionizable group with a pK value in this region, it follows that the protonation of a group on the enzyme–NADPH complex is essential for activity. The results of the solvent perturbation experiments (Table I) indicate that this group is a neutral acid, but the ΔH_{ion} value of 11.3 ± 2.1 kcal/mol does not permit definitive identification of the ionizing group. The high magnitude of the value suggests that ionization is accompanied by a conformational change which may complicate the interpretation of the results obtained for solvent effects. The data are consistent, however, with the idea that there is at the active site of dihydrofolate reductase a carboxyl group which is responsible for binding, for catalysis, or for both and which has an elevated pK value because it is in a hydrophobic environment.

The involvement of a protonated carboxyl group in the binding of ligands to the enzyme is confirmed by the pH profiles for 2,4-diaminopteridine (DAP), which acts as a linear competitive inhibitor with respect to dihydrofolate, and for methotrexate, which functions as a competitive, slow, tight-binding inhibitor (Figure 2). Analysis of the data for these profiles yielded pK values of 6.3 and 6.6, respectively, which are similar to the higher pK value of 6.6 from the V/K profile. Thus, it may be concluded that a true pK value is obtained from the V/K profile. From the data for the variation of the pK_i values with pH, it cannot be implied that the ionizing group is involved solely with the binding of dihydrofolate, especially when the formation of tetrahydrofolate must involve protonation and hydride transfer. If the group were involved also with catalysis, its ionization should be observed in the V profile although the pK value need not be identical with those obtained from the V/K and pK_i profiles. The V profile (Figure 1) shows that maximum enzyme activity is achieved on protonation of a single ionizing group with a pK of 8.6. Such a result is in accord with the hypothesis that the ionization of the same group is being observed in the V/K and V profiles and that the shift in the pK value by 2 pH units is due to the formation of an ionic type of hydrogen bond between the protonated carboxyl group on the enzyme and dihydrofolate. The presence of substrate on dihydrofolate reductase would then hinder the release of a proton from the ternary complex. In this connection, it should be noted that folate derivatives and analogues undergo partial protonation on reaction with dihydrofolate reductase (Poe et al., 1976). It should be mentioned that there is a possibility that the group with a pK value of 8.6 in the V profile is not the same group that is observed to have a pK value of 6.6 in the V/K profile.

From the results of the present study, it would be predicted that the chemical mechanism for the reduction of the N-5 to C-6 double bond of dihydrofolate involves protonation of the N-5 nitrogen of dihydrofolate by a protonated carboxyl group and subsequent hydride transfer from NADPH to the C-6 carbon [cf. Benkovic (1980)]. Further, it might be argued that since the same protonated carboxyl group on the enzyme is involved with the binding of methotrexate, which does not undergo reaction, the substrate and inhibitor must be bound

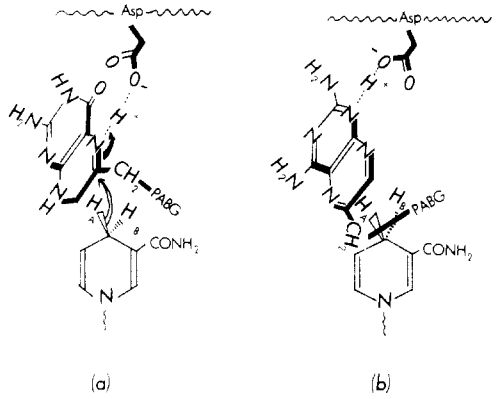

FIGURE 5: Partial structures to illustrate the different interactions of dihydrofolate (a) and methotrexate (b) with the dihydrofolate reductase–NADPH complex.

in different orientations. Such a difference could be accounted for on the basis that it is not the N-5 nitrogen of methotrexate that undergoes protonation, but rather the more basic N-1 (Poe, 1977), and that such a reaction does not facilitate reduction of either the N-5 to C-6 or the C-7 to N-8 double bond. The marked difference in the strength of interaction of methotrexate and 1-deazamethotrexate with an enzyme–NADPH complex emphasizes the importance of the N-1 nitrogen for the binding of methotrexate (Williams et al., 1980).

The concept that the combination of folate derivatives and analogues involves their interaction with a protonated carboxyl group is supported by the results of X-ray studies on the ternary complex formed with NADPH, methotrexate, and the enzyme from *Lactobacillus casei* (Matthews et al., 1978). These show that the N-1 nitrogen of methotrexate is close to an aspartate residue. Since this and other residues concerned with substrate binding are highly conserved in dihydrofolate reductases, it may be considered that an aspartate residue of the enzyme from *S. faecium* is involved with the binding of methotrexate and the reduction of dihydrofolate. Since this residue lies in a hydrophobic crevice of the enzyme from *E. coli*, its β-carboxyl group would be expected to have an elevated pK, relative to that for a carboxyl group exposed to solvent, which could well be in the vicinity of pH 6.6. The three-dimensional structure of an enzyme–dihydrofolate complex has not been observed, and thus, there is no direct demonstration that in such a complex there would be interaction between an aspartate residue and the N-5 of dihydrofolate. But it is clear that dihydrofolate is not bound in the same way as is methotrexate for then the hydride transfer from C-4 of the nicotinamide ring of NADPH would give rise to tetrahydrofolate with the R rather than the observed S configuration at the C-6 position (Armarego et al., 1980). Model building studies show that the correct stereochemistry of the reaction can be achieved by rotating the pteridine ring of methotrexate so as to allow the interaction of the N-5 with aspartate while leaving the C-4 of the nicotinamide moiety of NADPH and the C-6 of the pteridine ring in close proximity (Figure 5). Although there is a difference in the binding of methotrexate and dihydrofolate, it remains to be determined if such a difference is found only with slow, tight-binding inhibitors or if it occurs with all classes of inhibitors (Williams & Morrison, 1979).

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Histidine at the Active Site of *Neurospora* Tyrosinase†

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ABSTRACT: The involvement of histidyl residues as potential ligands to the binuclear active-site copper of *Neurospora* tyrosinase was explored by dye-sensitized photooxidation. The enzymatic activity of the holoenzyme was shown to be unaffected by exposure to light in the presence of methylene blue; however, irradiation of the apoenzyme under the same conditions led to a progressive loss of its ability to be reactivated with Cu^{2+}. This photoinactivation was paralleled by a decrease in the histidine content whereas the number of histidyl residues in the holoenzyme remained constant. Copper measurements of photooxidized, reconsituted apoenzyme demonstrated the loss of binding of one copper atom per mole of enzyme as a consequence of photosensitized oxidation of three out of nine histidine residues. Their sequence positions were determined by a comparison of the relative yields of the histidine containing peptides of photooxidized holo- and apotyrosinases. The data obtained show the preferential modification of histidyl residues 188, 193, and 289 and suggest that they consitute metal ligands to one of the two active-site copper atoms. Substitution of copper by cobalt was found to afford complete protection of the histidyl residues from being modified by dye-sensitized photooxidation.

Tyrosinase (monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) is a copper-containing monooxygenase which occurs widespread in microorganisms, plants, and animals (Lerch, 1981). The enzyme catalyzes the ortho hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones, leading to melanin pigments and other polyphenolic compounds (Mason, 1965). The enzymes from the common mushroom *Agaricus bispora* and *Neurospora crassa* have been shown to contain a copper pair at the active site which upon reduction of Cu^{2+} to Cu^{1+} binds oxygen reversibly (Schoot Uiterkamp & Mason, 1973; Makino et al., 1974; Deinum et al., 1976; Jolley et al., 1974; Lerch, 1976). In a recent resonance Raman study of *Neurospora* oxytyrosinase, the coordinated oxygen was shown to be bound as peroxide (Eickman et al., 1978), as was previously reported for the oxygen transporting hemocyanins (Freedman et al., 1976). In contrast to the rapidly increasing information on the involvement of the metal in the binding and activation of oxygen in these copper proteins (Eickman et al., 1979; Brown et al., 1980; Larrabee & Spiro, 1980; Himmelwright et al., 1980), the knowledge of the part played by the protein moiety especially with regard to the metal ligands and the active-site residues is still limited. Although a number of investigations implicate the involvement of histidyl residues as metal ligands of the active-site copper (Salvato et al., 1974; Tallandini et al., 1975; Engelborghs & Lontie, 1973; Gutteridge et al., 1977), progress has been hampered by the complexity and molecular heterogeneity of these molecules. In this report, the residues of the active-site region of *Neurospora* tyrosinase were identified by chemical modification employing dye-sensitized photooxidation. In conjunction with the recently determined primary structure of *Neurospora* tyrosinase (Lerch, 1978), the data suggest the involvement of histidyl residues 188, 193, and 289 in the binding of one of the two copper atoms of this monooxygenase.

Materials and Methods

Neurospora crassa wild-type strain (FGSC 320) producing the thermolabile form of tyrosinase was grown according to Horowitz et al. (1970). The enzyme from cycloheximide-derepressed cultures was isolated as described previously (Lerch, 1976). The specific activity of the purified enzyme was 1200 ± 100 units/mg as measured according the Fling et al. (1963). Protein concentration was determined by using the extinction coefficient $A_{280}^{1\%,1cm} = 22$ (Lerch, 1976). Amino acid analyses were performed after hydrolysis of peptide samples in 6 N HCl in sealed evacuated tubes for 22 h at 110 °C on a Durrum (Model D-500) amino acid analyzer. Tryptophan was determined by acid hydrolysis with 4.0 M methanesulfonic acid + 0.2% tryptamine (Inglis et al., 1976). Dye-sensitized photooxidation of holo- and apotyrosinases was carried out according to Forman et al. (1973). The reaction mixtures were exposed to a 250-W lamp of an ordinary slide projector at a distance of 30 cm. At given times, samples were withdrawn for activity measurements and amino acid analysis.

Apotyrosinase was prepared by treating the holoenzyme (100 μM with potassium cyanide (100 mM) for 2 h at 4 °C in 0.1 M sodium phosphate, pH 8.5, and subsequent gel filtration on Sephadex G-25 in 50 mM sodium phosphate, pH 7.5. This procedure resulted in apotyrosinase samples still containing 0.1–0.2 g-atom of copper per mol of enzyme, a value found to be independent on the residual enzymatic activity (0.1–1% of the native enzyme). Apotyrosinase was reconstituted by incubating the apoenzyme with a 20-fold molar excess of $CuSO_4$ for 20 h at 4 °C. The specific activity of the reconstituted enzyme was between 80% and 90% of the native enzyme. Prior to enzymatic digestion, holo-and apotyrosinases were freed from methylene blue by gel filtration on Sephadex G-25 in 25 mM ammonium acetate and lyophilized. The copper content of holo- and apotyrosinases was determined by atomic absorption spectrometry (Instrumen-

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